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Karyotype, C-banding and AgNORs of two endemic leuciscine fish, *Pseudophoxinus crassus* (Ladiges, 1960) and *P. hittitorum* Freyhof & Özulug, 2010 (Teleostei, Cyprinidae)

Sevgi Unal[1], Muhammet Gaffaroğlu[2], Muradiye Karasu Ayata[2], Eşref Yüksel[1]

1 *Gazi University, Science Faculty, Department of Biology, Ankara, Turkey* **2** *Ahi Evran University, Science and Art Faculty, Department of Biology, Kırşehir, Turkey*

Corresponding author: *Sevgi Ünal* (sevgiunal@ymail.com)

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Abstract

The genus *Pseudophoxinus* Bleeker, 1860 is found in a wide range of habitats in central Anatolia, but it is not well known from a cytogenetic aspect. In this study the first karyotypic description of the spring minnows *Pseudophoxinus crassus* (Ladiges, 1960) and *P. hittitorum* Freyhof & Özulug, 2010 by means of conventional methods (Giemsa staining, C-banding, silver nitrate impregnation (Ag-NORs)) was performed. Both species are endemic and have restricted distributions in Central Anatolia. *P. crassus* and *P. hittitorum* have the same diploid chromosome number, 2n = 50, patterns of distribution of constitutive heterochromatin (CH), and localization of nucleolus organizer regions (NORs), but differ in their karyotypic formulae (KFs). The C-banding technique revealed clear pericentromeric blocks of CH in many chromosomes; Ag-NORs treatment revealed consistent positive signals at the end of the short arms of a submetacentric chromosome pair, likely homologous in both species. The karyotypic differences found between these species can be used for their taxonomical study.

Keywords

Karyotype, C-banding, NOR-phenotype, Leuciscinae, cytotaxonomy

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Introduction

Spring minnows of the cyprinid genus *Pseudophoxinus* Bleeker, 1860 are distributed from Central Anatolia east to Azerbaijan and South to Israel (Freyhof and Özuluğ 2010). The genus belongs to the subfamily Leuciscinae, the major element of the Anatolia cyprinid fauna. Leuciscinae fishes include 54 species belonging to 17 genera in Anatolia, of which 26 species and subspecies are endemic. With 19 species recognized in Turkey, *Pseudophoxinus* is one of the most species-rich genera with a great number of the endemic species (Bogutskaya 1997, Freyhof and Özuluğ 2006, Bogutskaya et al. 2007, Karasu et al. 2011, Küçük et al. 2012, Küçük and Güçlü 2014). Species of this genus are found in a wide range of habitats in central Anatolia (Hrbek et al. 2004). According to IUCN, a significant point about the herein studied species is the fact that *P. crassus* and *P. hittitorum* are endangered (EN) species and their population trends are decreasing (IUCN 2014a; IUCN 2014b).

Karyotypic data for the genus are available only for *P. antalyae* Bogutskaya, 1992 and *P. firati* Bogutskaya, Küçük & Atalay, 2007 (Table 1). In both species a karyotype with 2n = 50 was revealed, indicating a conserved karyotypic evolution in relation to the diploid number (Ergene et al. 2010, Karasu et al. 2011). Thus, cytogenetic data for *Pseudophoxinus* are insufficient, and further study is needed to evaluate karyological characteristics of the genus, to improve the taxonomic identification of these fish, and to understand the evolutionary trends in this taxon (Yüksel and Gülkaç 1992).

The aim of this study is to describe the karyotypes of *P. crassus* and *P. hittitorum*, including identification of CH blocks and NORs by conventional cytogenetic techniques (Giemsa staining, C-banding, and Ag impregnation).

Material and methods

Specimens were captured by electrofishing in two distinct localities during the summer-autumn, 2012 and spring-summer, 2013. Three males and two females of *P. crassus* were collected in Cihanbeyli-İnsuyu spring (38°42'N, 32°45'E) and four females and four males of *P. hittitorum* in Beyşehir-Eflatunpınarı spring (37°52'N, 31°34'E). Specimens were transported alive to the laboratory and kept in well-aerated aquaria until analysis was performed. Chromosome spreads were obtained using standard kidney protocol (Collares-Pereira 1992). Chromosomes were stained with 4% Giemsa solution (pH = 6.8). C-bands were obtained according to Sumner technique (Sumner 1972). Silver impregnation to detect NORs followed the method of Howell and Black (1980).

The chromosome slides were observed by 100× objective with immersion oil and photographed using a Leica DM 3000 research microscope. AKAS software was used to take pictures of the metaphase plates. Measurements of chromosomes were performed by digital caliper from each individual and karyotypes were prepared manually. Chromosomes were arranged in decreasing size order and classified according to their arm ratios (Levan et al. 1964) in three categories: metacentric (M), submetacentric

Table 1. Cytogenetic data available for the genus *Pseudophoxinus*.

Species	Locality	2n	Karyotypic formula	FN	NOR	C-band	Reference
P. antalyae	Berdan River	50	16M+14SM+12ST+8A	92	1 pair *st.* *p* terminal	several	Ergene et al. 2010
P. firati	Tohma Creek	50	38M-SM+12ST	88	2 pairs *sm-st.* *p* terminal	6 pairs	Karasu et. al. 2011
P. crassus	İnsuyu Spring	50	12M+30SM+8ST-A	92	1 pair *sm* *p* terminal	several	Present study
P. hittitorum	Beyşehir Spring	50	14M+26SM+10ST-A	90	1 pair *sm* *p* terminal	several	Present study

2n: diploid number; FN: fundamental number; NOR: nucleolus organizer regions type; M: metacentric; SM: submetacentric; ST: subtelocentric; A: acrocentric; *p* short arm.

(SM) and subtelocentric to acrocentric (ST-A). To determine the fundamental number (FN), M and SM chromosomes were considered as bi-armed whereas those of group ST/A as uni-armed.

Results

243 metaphase plates were examined for *P. crassus* and 266 metaphase plates – for *P. hittitorum*. For *P. crassus* the percentage of the finding of 50 chromosomes was 81.50%. Other percentages were: for 49 chromosomes – 14.45%, for 48 chromosomes – 2.70%, for 47 chromosomes – 1.35%. For *P. hittitorum* the percentage of the finding of 50 chromosomes was 80.00%. Other percentages were: for 49 chromosomes – 13.50%, for 48 chromosomes – 3.00%, for 47 chromosomes – 2.30% and for 46 chromosomes – 1.20%. Therefore it was considered that the analyzed individuals of *P. crassus* and *P. hittitorum* had the same diploid numbers 2n = 50, but differed in their karyotypic formulas (KFs), which were 12 M + 30 SM + 8 ST-A (FN = 92) for *P. crassus* and 14 M + 26 SM + 10 ST-A (FN = 90) for *P. hittitorum*, respectively (Fig. 1). No sex chromosomes were identified for either species.

C-banding revealed the presence of the blocks of constitutive heterochromatin at the pericentromeric regions of many chromosome pairs in both species (Fig. 2).

The NORs were localized near to the secondary constriction on the short arm of a SM chromosome pair in both species (Fig. 3).

Discussion

P. crassus and *P. hittitorum* karyotypes demonstrated the general pattern described for most Leuciscinae that have the chromosome number (2n = 50), but their KFs differed. This is consistent with most other species of the genus *Pseudophoxinus*, which share 2n = 50 and differ in their KFs (Ergene et al. 2010, Karasu et al. 2011). The chromosome

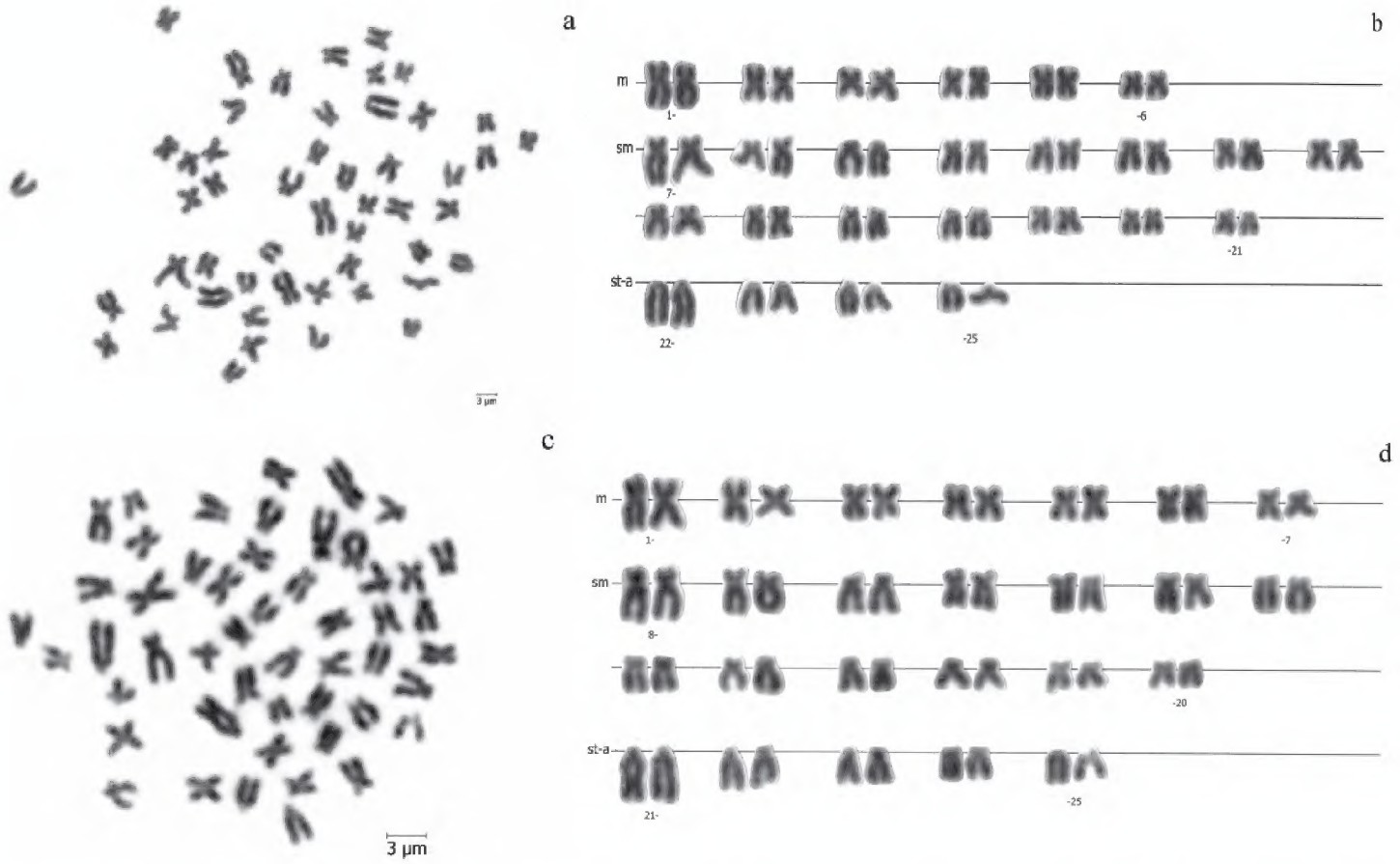

Figure 1. a Giemsa stained metaphase and **b** corresponding karyotype of *P. crassus* from Cihanbeyli stream **c** Giemsa stained metaphase and **d** karyotype of *P. hittitorum* from Beyşehir drainage. Scale bar = 3 µm.

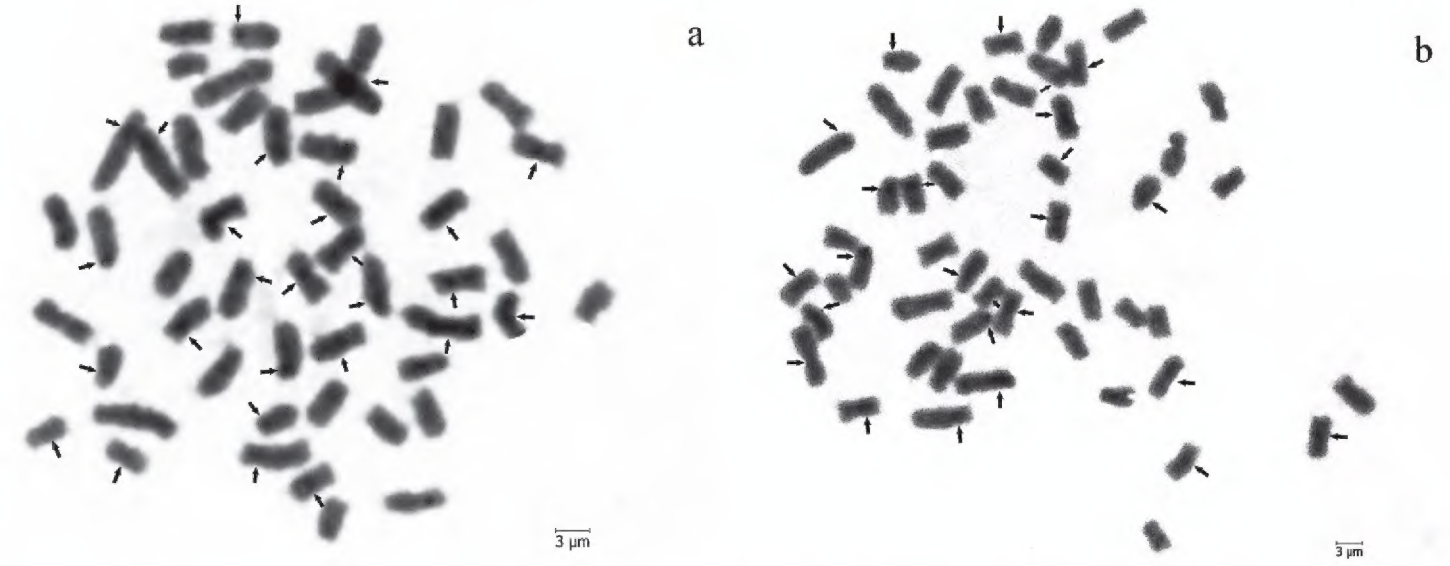

Figure 2. Metaphase spreads of (**a**) *P. crassus* and (**b**) *P. hittitorum* with C-banding. Arrows show CH regions. Scale bar = 3 µm.

sets of leuciscine cyprinids are characterized mainly by bi-armed (meta- and submetacentric) compared to the uni-armed (subtelo- and acrocentric) elements as observed in *P. crassus* and *P. hittitorum*. A large subtelocentric/acrocentric chromosome pair is considered as a cytotaxonomic marker for the subfamily Leuciscinae (Rab and Collares-Pereira 1995, Rab et al. 2008) and it is also present in both analysed species. However, cyprinid sex chromosomes appear to have remained morphologically undifferentiated (Sola and Gornung 2001). *P. crassus* and *P. hittitorum* also display the cyprinid characteristics mentioned above.

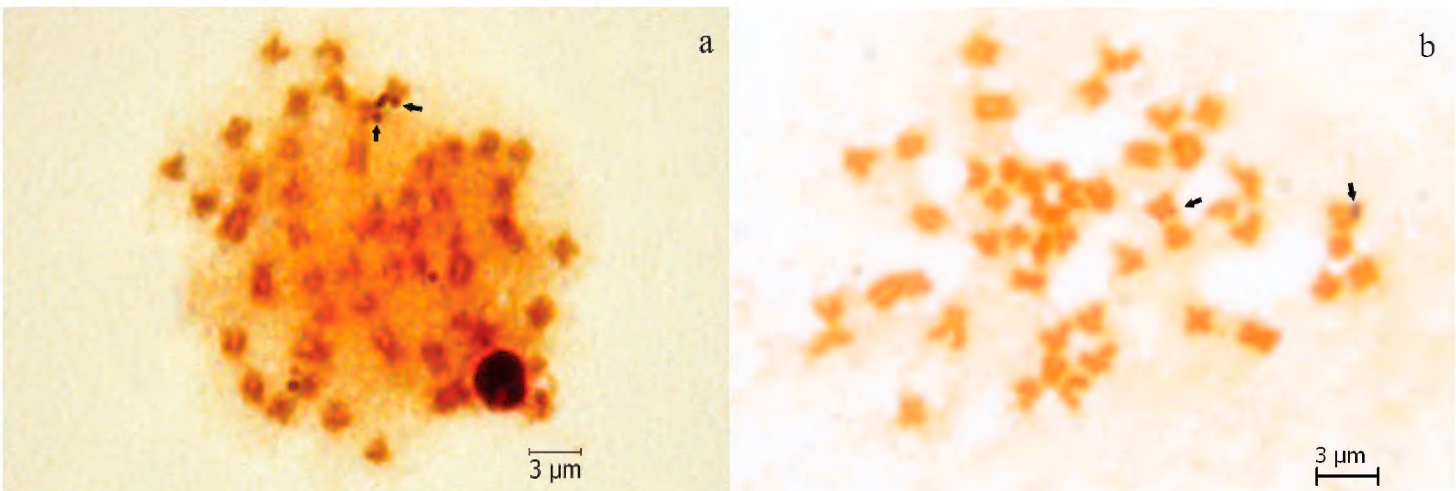

Figure 3. Metaphase spreads of (**a**) *P. crassus* and (**b**) *P. hittitorum* with Ag-NOR treatmets. Arrows show NORs. Scale bar = 3 µm.

C-bands identify regions of constitutive heterochromatin, which contain transcriptionally inactive highly repetitive DNA sequences (Gold et al. 1990). The difference in heterochromatin localization can be used as cytogenetic marker for the differentiation of species and for the reconstruction of chromosome evolution in the taxa (Gaffaroğlu and Yüksel 2009). In *P. crassus* and *P. hittitorum* C-positive blocks were pericentromeric, as in the *P. antalyae* (Ergene et al. 2010) and *P. firati* (Karasu et al. 2011). It was shown, that other studied Leuciscinae species as *Acanthobrama marmid* Heckel, 1843 (Gaffaroğlu and Yüksel 2009), *Squalius anatolicus* (Bogutskaya, 1997) (Ünal 2011) and *S. lucumonis* (Bianco, 1983) (Rossi et al. 2012) also have CH blocks on the pericentromeric regions. This pattern is conserved in Neotelostei as a whole, and also in all the Leuciscine genera examined to date (Collares-Pereira and Rab 1999, Boron et al. 2009, Rossi et al. 2012).

The number and location of NORs have been used as chromosome markers in fish cytotaxonomy (Pereira et al. 2012, Rossi et al. 2012, Nabais et al. 2013). The NORs located on a medium-sized SM chromosome pair corresponds to those observed in many of the leuciscines analyzed (Bianco et al. 2004). In spite of the many exceptions reported in Leuciscinae species from both Eurasia and North America (Pereira et al. 2009, Rossi et al. 2012), a single pair of NOR-carrying chromosome is considered as an ancestral character in this lineage (Rab and Collares-Pereira 1995, Rab et al. 2007). Within the genus *Pseudophoxinus*, a single NOR-bearing chromosome pair as in *P. crassus* and *P. hittitorum*, was observed in *P. antalyae* (Ergene et al. 2010) whereas multiple NOR-carrying chromosomes were detected in *P. firati* (Karasu et al. 2011). Although NORs are usually located on the short arms of chromosomes, sometimes they can be seen on the long arms of metacentric and acrocentric chromosomes (Rab and Collares-Pereira 1995, Rab et al. 1996). Furthermore, NORs can be seen between telomeres and centromeres (Amemiya and Gold 1988). Generally, the NOR-phenotype is observed at the terminal on short arms of mid-sized A-ST chromosomes (Takai and Ojima 1992), and rarely at the terminal on short arms of mid-sized SM chromosomes (Gold et al. 1988, Magtoon and Arai 1993) like in *P. crassus* and *P. hittitorum*. Conversely to what was reported for some others leuciscin cyprinids (Ünal

2011), no NOR polymorphism was observed in the specimens from our study. Further, there is no report of any variation in NORs' phenotype in all analyzed individuals of the genus *Pseudophoxinus* (Ergene et al. 2010, Karasu et al. 2011). Thus the karyotypes of these species conserved plesiomorphic condition that is confirmed by present study.

In conclusion, the karyotypic differences and CH and NOR localizations found in the two *Pseudophoxinus* species studied herein can be used as a cytogenetic comparison data.

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